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MONOPHYLY OF THE MOITESSIERIIDAE BOURGUIGNAT, 1863 (CAENOGASTROPODA: TRUNCATELLOIDEA)

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ABSTRACT: The family Moitessieriidae is poorly known, as its members, inhabiting exclusively subterranean waters, are often known only from few minute, empty shells. Molecular studies on their relationships confirmed the distinctness of this family. Their monophyly, however, remained doubtful, since the Moitessieriidae did not form a distinct clade in the phylogenetic tree based on the most commonly applied mitochondrial cytochrome oxidase subunit I (COI), and the representative of the family Cochliopidae occupied a position among the moitessieriid clades. In the present paper two new nuclear loci, namely histone H3 gene and ribosomal internal transcribed spacer ITS2, have been applied to resolve the status of the Moitessieriidae. The resulting phylogenies show the Moitessieriidae as a distinct, well supported clade, confirming thus their monophyly.

KEY WORDS: Moitessieriidae, molecular phylogeny, COI, H3, 18S, ITS2

INTRODUCTION

The family Moitessieriidae Bourguignat, 1863 is probably the least studied group of the European Truncatelloidea. It includes minute dioecious gastropods (Figs 1–11) exclusively inhabiting subterranean waters, including thermal ones (SKET & VELKOVRH 1981). For most species only empty shells are known. Hence, dozens of nominal species have been described without anatomical and molecular data (e.g. GLÖER 2002), and their distinctness as well as their relationships remain unknown. Comparative molecular data have only recently begun to accumulate (WILKE et al. 2001, 2013, RICHLING et al. 2016, ANGYAL et al. 2018, HOFMAN et al. 2018), but it is already evident that the morphology-based levels of endemism are overestimated in the Moitessieriidae (FALNIOWSKI et al. 2014).

The morphology and anatomy of the soft parts are still poorly studied. The detailed anatomy of *Bythiospeum* Bourguignat, 1882 was described and illustrated by HAASE (1995). Some anatomical

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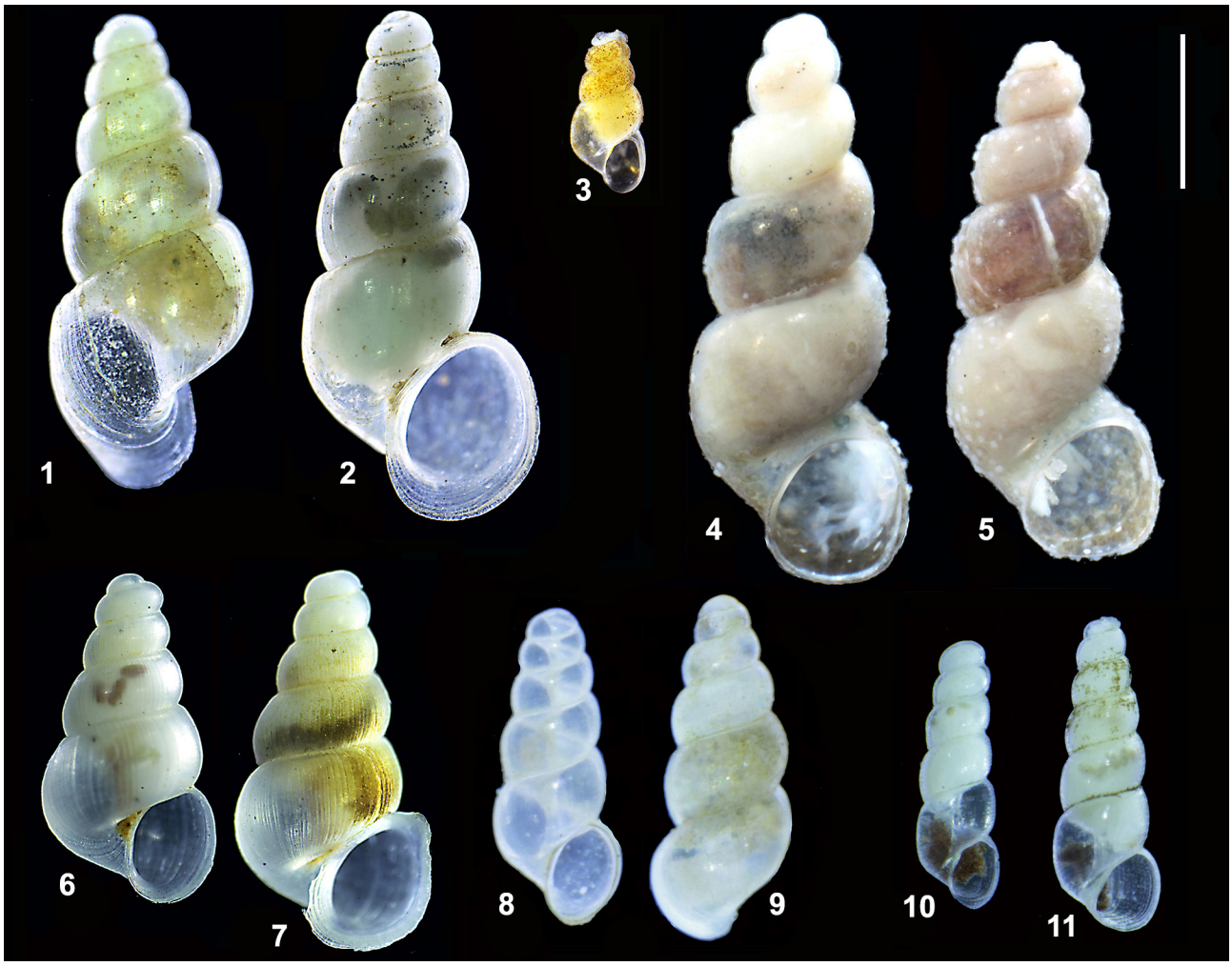


Figs 1–11. Shells of the Moitessieriidae: 1–2 – *Paladilhiopsis grobbeni* Kuščer, 1928; 3 – *Bythiospeum acicula* (Hartmann, 1821); 4–5 – *Iglica hellenica* Falniowski et Sarbu, 2015; 6–7 – *Costellina turrita* Kuščer, 1933; 8–9 – *Iglica* cf. *forumjuliana* (Pollonera, 1887); 10–11 – *Moitessieria* sp. (after HOFMAN et al. 2018, except 3); scale bar 1 mm

data on the Moitessieriidae were contributed also by BOLE (1961, 1970), GIUSTI & PEZZOLI (1980) (anatomy of *Iglica* Wagner, 1927 and *Paladilhiopsis* Pavlović, 1913), BERNASCONI (1990, 1994), BOETERS & GITTENBERGER (1990), BODON & GIUSTI (1991), BOETERS (1998), SZAROWSKA (2006) and NIERO & PEZZOLI (2016), summarised by WILKE et al. (2013). The character states unique for the Moitessieriidae are: pyriform metapodial tentacle, and intestine with a long, wide and loose loop around the style sac. The character states of moitessieriids that are uncommon among the Truncatelloidea are: smooth protoconch; all soft parts unpigmented; ctenidium reduced or absent; and osphradium small, less than twice as long as broad (HOFMAN et al. 2018). The distinctness of the Moitessieriidae has been questioned: BODON & GIUSTI (1991) considered its representatives to belong to the Hydrobiidae.

Molecular data (WILKE et al. 2001, 2013, HOFMAN et al. 2018) have confirmed the distinctness of the Moitessieriidae. However, HOFMAN et al. (2018) found that the family Cochliopidae was nested among the moitessieriid clades based on a cytochrome oxidase subunit I (COI) tree and, despite the low associated bootstrap values, the monophyly of the family Moitessieriidae was not supported. This was in contrast to findings of WILKE et al. (2013), who reported its monophyly; it should be noted, though, that the family representatives failed to form a distinct clade, and neither *Iglica* nor *Paladilhiopsis* were included in the phylogenetic analysis. In the COI tree of HOFMAN et al. (2018) there was a well-supported clade containing all the Balkan *Paladilhiopsis* and *Iglica hellenica* Falniowski et Sarbu, 2015, another well supported clade of *I.* cf. *gracilis* (Clessin, 1882) (both formed a weakly supported clade), a third well-supported clade of the Cochliopidae, and the fourth well-supported clade grouping together all members of the "real" *Bythiospeum*. These four clades formed an unresolved polytomy together with *Sardopaladilhia* Manganelli, Bodon, Cianfanelli, Talenti et Giusti, 1998 and *Moitessieria* Bourguignat, 1863. Bootstrap supports were very low, thus the Moitessieriidae did not form a well supported clade. Unfortunately, as only the

COI sequences could be retrieved from GenBank and no *Bythiospeum* material was available for sequencing any nuclear loci at that time, the problem remained unresolved. The aim of the present paper is to test the monophyly of the Moitessieriidae by investigating nuclear loci.

MATERIAL AND METHODS

Specimens of *Bythiospeum acicula* (Hartmann, 1821) (Fig. 3) were collected in Switzerland, Kollbrunn, site RA204, Töss river, 47°27'25.7"N, 8°46'09.9"E. The Bou-Rouch method (Bou & Rouch 1967) was used to sample a gravel bar of the river. At the same place also the recently described interstitial amphipod species *Niphargus tonywhitteni* was found (Fišer et al. 2018). The collection localities of all the other moitessieriid taxa have been described in Hofman et al. (2018). Samples were preserved in 96% ethanol. All the techniques of fixation, DNA extraction and PCR conditions were exactly the same as in Szarowska et al. (2016a, b) for mitochondrial cytochrome oxidase subunit I (COI), nuclear 18S ribosomal RNA (18S), and nuclear histone H3 (H3). For internal transcribed spacer 2 (ITS2) the following primers were used: NEWS2-F 5'-TGTGTCGATGAAGAACGCAG-3'; and ITS2-RIXO 5'-TTCTATGCTTAAATTCAGGGG-3'; Almeyda-Artigas et al. 2000). The PCR conditions (Phusion High-Fidelity DNA Polymerase) were as follows: initial denaturation step of 1 min at 98 °C, followed by 36 cycles of 10 s at 98 °C, 10 s at 48 °C, 20 s at 72 °C, and a final extension of 5 min at 72 °C. The saturation test (Xia et al. 2003) for the coding genes was performed using DAMBE (Xia 2013). Sequences were initially aligned in the MUSCLE (Edgar 2004) algorithm integrated in MEGA 6 (Tamura et al. 2013). Names of taxa used for phylogenetic inference, GenBank accession numbers of sequences and their references are provided in Table 1. Phylogenetic inference was run applying maximum likelihood technique with RAxML (Stamatakis 2014), estimating with RAxML appropriate parameters of the GTR+I model for each partition of concatenated sequences, in the coding ones each codon position treated in the same way.

RESULTS AND DISCUSSION

The identification of our specimen of *Bythiospeum* needs some explanation. In the Swiss faunistic list (Centre Suisse de Carthographie de la Faune CSCF) *Bythiospeum haeussleri* (Clessin, 1910) is recorded from the same region as our *Bythiospeum*. However, our sequence is identical with the ones from GenBank, representing five nominal species (Fig. 12). According to the ICZN, namely the priority rule, the name *Bythiospeum acicula* (Held, 1838) is appropriate for this taxon. According to Richling et al. (2016), *B. haeussleri*, known from Switzerland, belongs to their clade 3, and *B. acicula*, inhabiting Germany NE of our Swiss locality, belongs to their clade 1, their sequences are markedly different one from another. *B. acicula* has not been recorded from Switzerland so far, but Richling et al. (2016) suggest that its range could also reach Switzerland.

Finally, we obtained 16 sequences of 18S (286 bp, GenBank Accession numbers MK629727-MK629742), 16 sequences of ITS2 (310 bp, GenBank Accession numbers MK629747-MK629762), three sequences of H3 (310 bp, GenBank Accession numbers MK609534-MK609536) and one of COI (488 bp, GenBank Accession number MK609537). In both coding loci the tests of Xia et al. (2003) revealed no saturation. The cytochrome oxidase tree including our specimen of *Bythiospeum* (Fig. 12) showed again the clade which consisted of the representatives of the Cochliopidae Tryon, 1866, within the polytomy formed by three clades of the Moitessieriidae (*Moitessieria/Sardopaladilhia*, *Bythiospeum* and *Iglica/Paladilhiopsis*), as also in Hofman et al. (2018). In the tree of 18S (not shown) the Moitessieriidae were far from the Cochliopidae, but all the bootstrap supports were low. In the tree of nuclear H3 (Fig. 13) the Moitessieriidae formed a distinct, well supported clade (bootstrap support 74%). Interestingly, *Bythiospeum* was close to *Iglica*, not to *Paladilhiopsis*, which confirmed the results of Hofman et al. (2018), but not those of Boeters (1998). The latter author synonymised *Paladilhiopsis* with *Bythiospeum*, based on the similarity of the general structure of the female reproductive organs, i.e., a large bursa copulatrix situated at the proximal part of the albumen gland, which is markedly shortened. These character states were regarded as a result of parallelism by Hofman et al. (2018).

The tree from concatenated sequences of all nuclear loci (Fig. 14) grouped all Moitessieriidae taxa in one well supported clade (76%). The same applies to the tree from concatenated sequences of both, nuclear and mtCOI loci (support 71%). There are no strict rules concerning the significance levels of the bootstrap, but usually the values exceeding 70% are accepted as significant. Thus, we consider the Moitessieriidae as monophyletic, although closely related to Cochliopidae.

Table 1. Data obtained from GenBank Nucleotide database. Names of taxa used for phylogenetic analyses with their accession numbers and references are provided

Species	GenBank numbers		References for GenBank data	
	COI	H3	COI	H3
Agrafia wiktori Szarowska et Falniowski, 2011	JF906762	MG543158	Szarowska & Falniowski (2011)	Grego et al. (2017)
Alzoniella finalina Giusti et Bodon, 1984	AF367650		Wilke et al. (2001)	
Anagastina zetavalis (Radoman, 1973)	EF070616		Wilke et al. (2001)	
Avenionia brevis berenguieri (Draparnaud, 1805)	AF367638		Szarowska (2006)	
Belgrandiella kusceri (Wagner, 1914)	KT218511	MG551366	Falniowski & Beran (2015)	Osikowski et al. (2018)
Bithynia tentaculata (Linnaeus, 1758)	AF367643		Wilke et al. (2001)	
Bythinella austriaca (von Frauenfeld, 1857)	JQ639858		Falniowski et al. (2012b)	
Bythinella micherdzinskii Falniowski, 1980	JQ639854		Falniowski et al. (2012b)	
Bythiospeum acicula (Hartmann, 1821)	KU341350		Richling et al. (2016)	
Bythiospeum acutum (Geyer, 1904)	HM107120		Hirsch et al. (2010)	
Bythiospeum alzense Boeters, 2001	KU341354		Richling et al. (2016)	
Bythiospeum francomontanum Bernasconi, 1973	HM107128		Hirsch et al. (2010)	
Bythiospeum cf. *gebhardti* (H. Wagner, 1931)	KP296922		Angyal et al. (2018)	
Bythiospeum hungaricum (Soos, 1927)	KP296923		Angyal et al. (2018)	
Bythiospeum husmanni (C. Boettger, 1963)	HM107134		Hirsch et al. (2010)	
Bythiospeum pellucidum (Seckendorf, 1846)	HM107124		Hirsch et al. (2010)	
Bythiospeum quenstedti (Wiedersheim, 1873)	HM107113		Hirsch et al. (2010)	
Bythiospeum saxigenum (Geyer, 1905)	HM107125		Hirsch et al. (2010)	
Bythiospeum suevicum (Geyer, 1905)	HM107118		Hirsch et al. (2010)	
Dalmatinella fluviatilis Radoman, 1973	KC344541		Falniowski & Szarowska (2013)	
Daphniola louisi Falnowski et Szarowska, 2000	KM887915		Szarowska et al. (2014a)	
Ecrobia maritima (Milaschewitsch, 1916)	KJ406200	MG551322	Szarowska & Falniowski (2014b)	Grego et al. (2017)
Emmericia expansilabris Bourguignat, 1880	KC810060		Szarowska & Falniowski (2013a)	
Fissuria boui Boeters, 1981	AF367654		Wilke et al. (2001)	
Graziana alpestris (Frauenfeld, 1863)	AF367641		Wilke et al. (2001)	
Grossuana codreanui (Grossu, 1946)	EF061919		Szarowska et al. (2007)	
Hauffenia michleri Kuščer, 1932		KY087878		Rysiewska et al. (2017)
Heleobia dalmatica (Radoman, 1974)	JQ973022		Kroll et al. (2012)	
Heleobia dobrogica (Grossu et Negrea, 1989)	EU938131		Falniowski et al. (2008)	
Heleobia foxianensis (De Stefani, 1883)	JQ973023		Kroll et al. (2012)	
Heleobia maltzani (Westerlund, 1886)	KM213723	MK609534	Szarowska et al. (2014b)	this paper
Heleobia stagnorum (Gmelin, 1791)	JQ973024		Kroll et al. (2012)	
Heleobops carrikeri Davis et McKee, 1989	AF213347		Wilke et al. (2001)	

Table 1. continued

Species	GenBank numbers		References for GenBank data	
	COI	H3	COI	H3
Horatia klecakiana Bourguignat 1887	KJ159128		Szarowska & Falniowski (2014a)	
Hydrobia acuta (Draparnaud, 1805)	AF278808		Wilke et al. (2000)	
Iglica cf. *forumjuliana* (Pollonera, 1887)		MH721006	Hofman et al. (2018)	
Iglica cf. *gracilis* (Clessin, 1882)	MH720985-MH720989	MH721003-MH721004	Hofman et al. (2018)	
Iglica cf. *hauffeni* (Brusina, 1886)		MH720995	Hofman et al. (2018)	
Iglica hellenica Falniowski et Sarbu, 2015	KT825581	MH721007	Falniowski & Sarbu (2015)	Hofman et al. (2018)
Islamia zermanica (Radoman, 1973)	KU662362	MG551320	Beran et al. (2016)	Grego et al. (2017)
Lithoglyphus prasinus (Küster, 1852)	JX073651		Falniowski & Szarowska (2012)	
Littoridinops monroensis (Frauenfeld, 1863)	AF367644		Wilke et al. (2001)	
Littorina littorea (Linnaeus, 1758)	KF644330		Layton et al. (2014)	
Marstoniopsis insubrica (Küster, 1853)	AF322408		Falniowski & Wilke (2001)	
Moitessieria cf. *puteana* Coutagne, 1883	AF367635	MH721012	Wilke et al. (2001)	Hofman et al. (2018)
Montenegrospeum bogici (Pešić et Glöer, 2012)	KM875510	MG880218	Falniowski et al. (2014)	Grego et al. (2018)
Onobops jacksoni (Bartsch, 1953)	AF367645		Wilke et al. (2001)	
Paladilhiopsis cf. *absoloni* (A. J. Wagner, 1914)		MH721021		Hofman et al. (2018)
Paladilhiopsis blihensis (Glöer et Grego, 2015)		MH721015		Hofman et al. (2018)
Paladilhiopsis bosniaca (Clessin, 1910)		MH721020		Hofman et al. (2018)
Paladilhiopsis bosnica Bole, 1970		MH721021		Hofman et al. (2018)
Paladilhiopsis grobbeni Kuščer, 1928	MH720990-MH720991	MH721013	Hofman et al. (2018)	Hofman et al. (2018)
Paladilhiopsis maroskoi (Glöer et Grego, 2015)		MH721017		Hofman et al. (2018)
Paladilhiopsis turrita (Kuščer, 1933)	MH720992	MH721015	Hofman et al. (2018)	Hofman et al. (2018)
Paladilhiopsis gittenbergeri (A. Reischutz et P. L. Reischutz, 2008)	MH720993	MH721025	Hofman et al. (2018)	Hofman et al. (2018)
Pontobelgrandiella sp. Radoman, 1978	KU497024	MG551321	Rysiewska et al. (2016)	Grego et al. (2017)
Pseudamnicola sp. Paulucci, 1878		KT710579		Szarowska et al. (2016a)
Pyrgophorus platyrachis F. G. Thompson, 1968	AF367632		Wilke et al. (2001)	
Radomaniola curta (Küster, 1853)	KC011814		Falniowski et al. (2012a)	
Sadleriana fluminensis (Küster, 1853)	KF193067		Szarowska & Falniowski (2013b)	
Sardopaladilhia plagigeyerica Manganelli, Bodon, Cianfanelli, Talenti et Giusti, 1998	HQ623176		Wilke et al. (2013)	
Semisalsa dalmatica Radoman, 1974	AF367631		Wilke et al. (2001)	
Spurwinkia salsa (Pilsbry, 1905)	AF367633		Wilke et al. (2001)	
Tanousia zrmanjae (Brusina, 1866)	KU041812		Beran et al. (2015)	

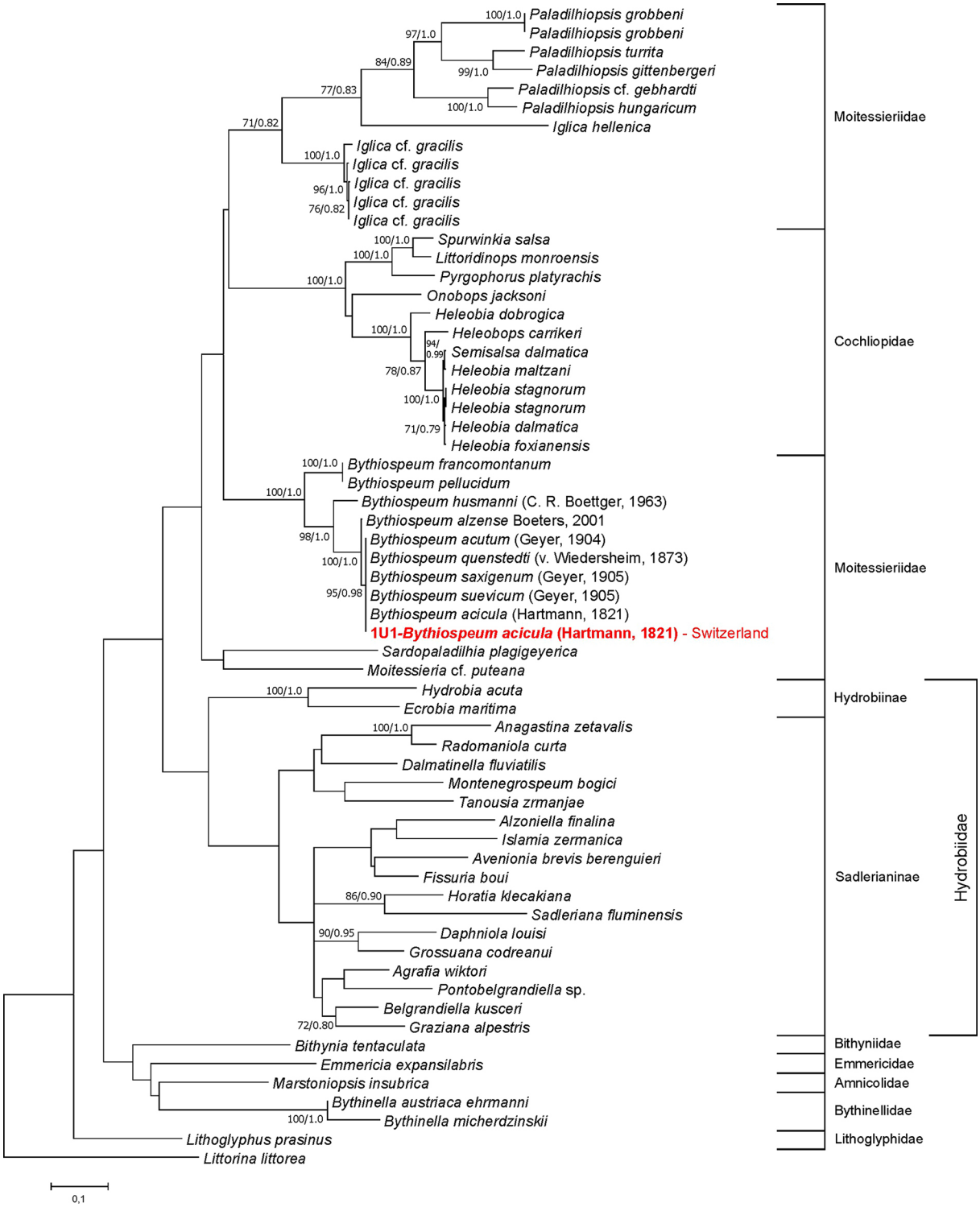


Fig. 12. Maximum likelihood tree of cytochrome oxidase subunit I (COI); bootstrap supports and Bayesian posterior probabilities given. New sequence marked by red bold

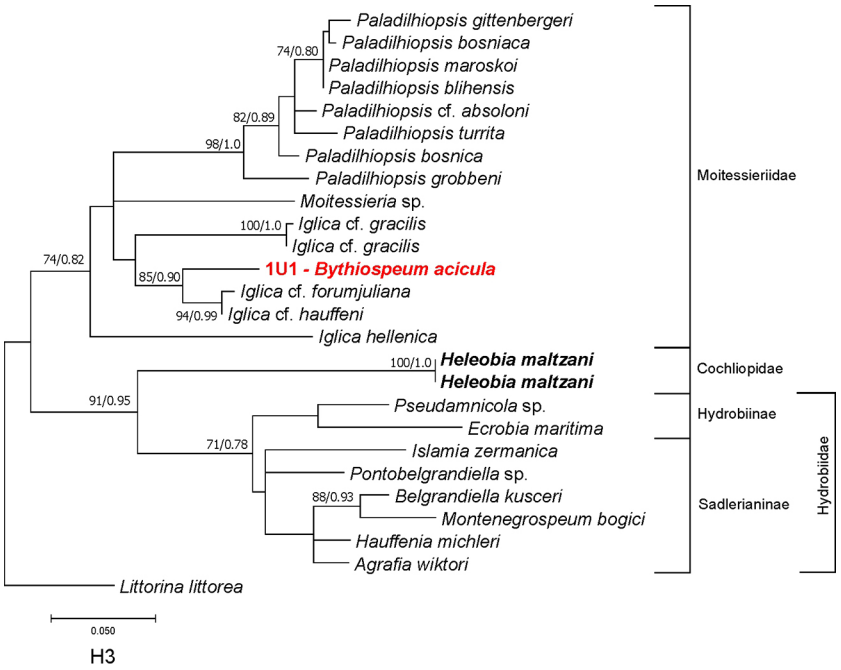
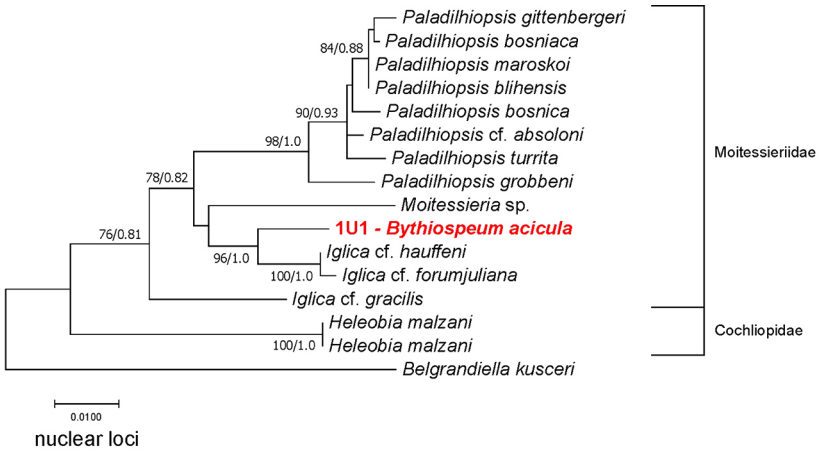


Fig. 13. Maximum likelihood tree of histone H3; bootstrap supports and Bayesian probabilities given. New sequences marked by bold



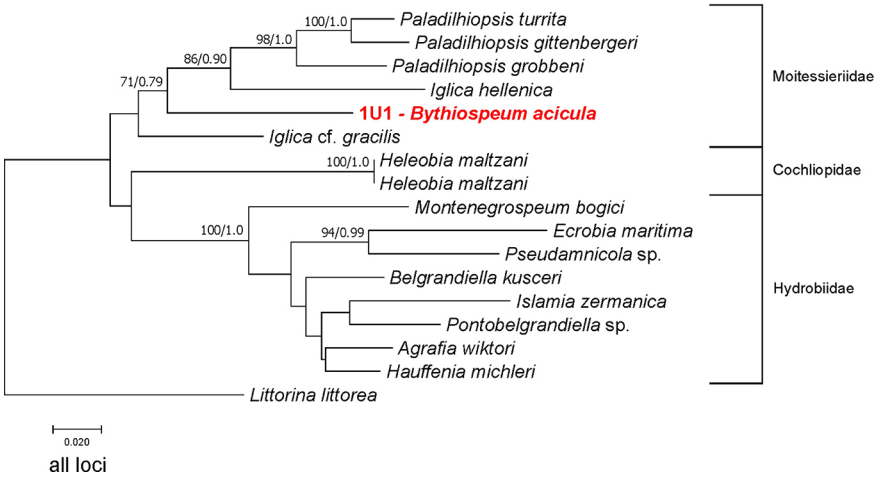


Fig. 14. Maximum likelihood tree of concatenated nuclear loci (including ITS2; above) and concatenated nuclear and mtCOI loci (below); bootstrap supports and Bayesian probabilities given above the branches. New sequences marked by bold

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